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EXPRESSION OF FLOTILLIN-1 ON *EIMERIA TENELLA* SPOROZOITES AND ITS ROLE IN HOST CELL INVASION

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ABSTRACT: Lipid rafts are detergent-resistant, liquid-ordered microdomains in plasma membranes that are enriched in cholesterol and sphingolipids and involved in intracellular signal transduction, membrane trafficking, and molecular sorting. In this study, we investigated the possibility that lipid rafts on *Eimeria tenella* sporozoites may act as platforms for host cell invasion. Flotillin-1, a resident protein of lipid rafts, was identified on *E. tenella* sporozoites and was prominently expressed at the apex of the cells, a region mediating host cell invasion. Pretreatment of sporozoites with antibody against flotillin-1 blocked parasite invasion. Furthermore, the anticoccidial drug, monensin, disrupted the localization of flotillin-1 within raft structures resulting in loss of invasion. We conclude that *Eimeria* sporozoites utilize lipid rafts containing flotillin-1 for internalization into host cells.

Eimeria tenella is an intracellular protozoan parasite that invades cecal epithelial cells, where it multiplies, leading to the development of coccidiosis. The molecular mechanisms through which parasites infect host cells are not well known. During the last few years, studies on the invasion process by intracellular protozoans have provided information on the role that cellular membranes play during parasite internalization (López-Bernad et al., 1996; Entzeroth et al., 1998; Brown, 2003). Increasing evidence suggests that host cell invasion by apicomplexan parasites involves a cascade of events, including parasite motility, attachment to the host cell surface, signal transduction, secretion of apical vesicles, and formation of the parasitophorous vacuole (Dubremetz et al., 1998; Bumstead and Tomley, 2000; Opitz and Soldati, 2002).

Many proteins involved in signal transduction and vesicular trafficking are abundant in lipid rafts, such as Src-family tyrosine kinases, protein kinase C, heterotrimeric and small G proteins, tyrosine kinase receptors, and G-protein–coupled receptors (Anderson, 1998; Edidin, 2003). Lipid rafts are detergent-resistant, liquid-ordered microdomains in plasma membranes that are enriched in cholesterol and sphingolipids, and play critical roles in many biological processes, such as signal transduction, apoptosis, cell migration, synaptic transmission, organization of the cytoskeleton, and protein sorting (Ding et al., 2004). We hypothesized that *Eimeria* spp. rafts may act as platforms for conducting the variety of cellular functions involved in host cell invasion.

In an attempt to identify the parasite surface components involved in the interaction with the host cell, in this research we have focused on the rafts of *Eimeria tenella* that may be involved in the host cell invasion process. For this purpose, an antibody that specifically reacts with a resident lipid raft protein, flotillin-1, was used. Flotillin-1 is known as a key structural component and a marker of lipid rafts (Wakasugi et al., 2004).

Monensin is a biologically active polyether sodium- and potassium-selective carboxylic ionophore antibiotic produced by *Streptomyces cinnamonensis*. Monensin is often used in experimental research for its transmembrane ionophoric attributes (Foey et al., 1997; Marumoa and Wakabayashia, 2005). Although it has a limited antibacterial activity (Russell and Houlihan, 2003), the drug is used worldwide in veterinary medicine for improvement in feed efficiency in cattle and as a feed additive against coccidiosis in chickens, turkeys, quail, goats, and cattle. It has been proposed that monensin is a highly lipophilic polyether that accumulates in cell membranes and causes profound alterations in its lipid composition (Mollenhauer et al., 1990; Gordon and Lloyd, 1994; Brown and London, 2000). The anticoccidial activity of monensin and its ability to regulate the level of cell surface sphingolipids led us to study the effect of monensin on the rafts of sporozoites of *E. tenella* and the role of *E. tenella* lipid rafts during parasite invasion. The present study was conducted to determine, via immunofluorescence and immunoblotting, the expression and localization of flotillin-1 on sporozoites of *E. tenella*. In addition, evidence is provided that lipid rafts containing flotillin-1 are involved in host invasion.

MATERIALS AND METHODS

Experimental design

A monensin-sensitive strain of *E. tenella* was obtained from litter samples collected from broiler grower houses located in Zaragoza, Spain. It was cultivated from a single oocyst isolated by a micromanipulator (Eppendorf, Hamburg, Germany).

To study the effect of monensin on the expression of flotillin in *E. tenella* sporozoites, a monensin-resistant line was obtained from the above-mentioned strain following the procedure of Zhu et al. (1994). Purified sporozoites of both the parent strain (12×10^6) and the monensin-resistant line (12×10^6) were divided into 4 groups, groups 1 and 2 (parent strain) and groups 3 and 4 (resistant line), consisting of 6×10^6 sporozoites each. Those from groups 1 and 3 were incubated in phosphate-buffered saline (PBS), pH 7.2, whereas those from groups 2 and 4 were challenged with monensin at 0.3 μg/ml in PBS. Sporozoites were incubated at 41 C for 3 hr. Samples were then obtained to study sporozoite infectivity and flotillin-1 expression.

To examine the effect of anti–flotillin-1 antibody on the infectivity of *E. tenella* sporozoites, 2 groups (A and B) of 3×10^6 sporozoites each were suspended in PBS and treated as follows. Group A sporozoites were incubated with 30 μl of anti–flotillin-1 antibody for 30 min at 41 C. Group B sporozoites were incubated in normal rabbit IgG, instead of the antibody. After incubation, samples were obtained to study sporozoite infectivity.

Production of a monensin resistant *E. tenella* strain

Chicken were infected by oral inoculation with sporulated *E. tenella* oocysts into the crop. Fecal oocysts were isolated and sporulated according to the procedures described by Raether et al. (1995). Sporozoites were excysted and separated from the oocysts and sporocyst debris according to the procedures described by Zhu et al. (1994). To isolate a monensin-resistant parasite strain, purified sporozoites of the monensin-sensitive parent strain were suspended in PBS, treated with

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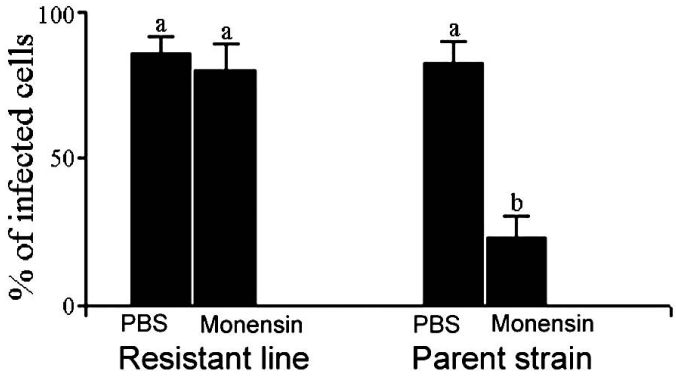

FIGURE 1. Flow cytometric quantification of sporozoite infectivity of the monensin-resistant *E. tenella* strain or the parent monensin-sensitive strain after incubation in PBS or monensin. Each bar represents the mean ± SD of the percentage of infected cells. Note the significant reduction in infectivity of the parent strain challenged with monensin. All values with different superscripts are significantly different by Duncan's multiple range test ($P < 0.05$).

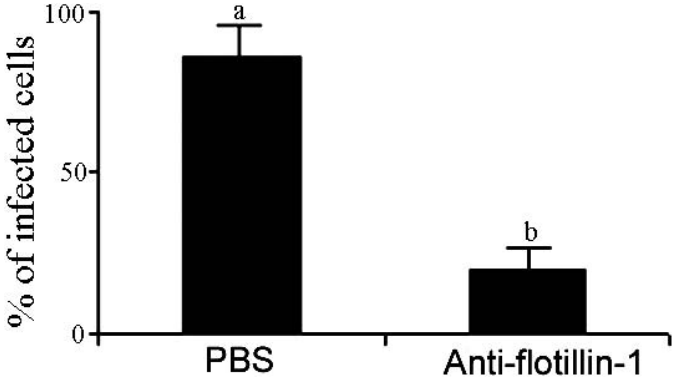

FIGURE 2. Effect of anti–flotillin-1 antibody on sporozoite infectivity. Each bar represents the mean ± SD of the percentage of infected cells after incubation of sporozoites with PBS or anti–flotillin-1 antibody. The different superscripts demonstrate significantly different infectivity ($P < 0.05$).

0.01 μg/ml monensin for 3 hr at 41 C, and washed 3 times with PBS. A minimum of 1×10^6 monensin-treated sporozoites were inoculated into 1-wk-old chickens that were maintained on a ration containing 50 ppm of monensin. On day 7 postinfection, oocysts were collected from the ceca and sporulated. The entire procedure was repeated 3 times with 0.02, 0.04, 0.08, 0.10, 0.20, 0.40, 0.60, and 0.80 μg/ml monesin in separate assays.

Determination of sporozoite infectivity

Chicken primary kidney cells (CPKC) were cultured on 12-mm round cover slips in 24-well cell culture plates with RPMI 1640 medium (Sigma, St. Louis, Missouri) containing 5% fetal bovine serum at 41 C in 5% CO_2. Sporozoites were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen, Carlsbad, California); the fluorescent sporozoites were added to CPKC cultures at 1×10^5 sporozoites/well and incubated for 5 hr at 41 C. The cells were trypsinized, washed with PBS, fixed with 2% paraformaldehyde in PBS for 10 min, washed, filtered through a 250-μm mesh screen, and analyzed by fluorescence-activated cell sorting using a FACScan instrument (BD Biosciences, San Jose, California) at 488 nm. Each assay was performed in triplicate.

Immunofluorescence analysis

Sporozoites were fixed in ethanol and incubated with normal serum-blocking reagent (Vector Laboratories, Burlingame, California) for 10 min at room temperature followed by anti–flotillin-1 antibody at a 1:100 dilution for 90 min. Sporozoites were washed 3 times in PBS, recovered by centrifugation, and incubated with fluorescein-conjugated goat anti-mouse IgG antibody (Dako Laboratories, Glostrup, Denmark) for 30 min. Fluorescent sporozoites were visualized with a BH-2 fluorescence microscope (Olympus, Hamburg, Germany). As a negative control, sporozoites were incubated with normal IgG instead of the primary antibody.

Immunoblot analysis

Extraction of proteins was performed following the procedure described in Nagao et al. (2002). Sporozoites (2×10^6) were suspended in 200 μl of 0.01 M Tris-HCl, pH 7.4, containing 0.15 M NaCl (TBS) and lysed with 800 μl of 1% Triton X-100 in TBS for 20 min on ice; the lysate was mixed with an equal volume of 80% sucrose in 0.2 M Na_2CO_3. The lysate was overlayed with 4 ml of 30% sucrose and 2 ml of 10% sucrose in TBS, and centrifuged for 17 hr at 230,000 *g* at 4 C. Thirty fractions (approximately 300 μl each) were collected from the top of the centrifuge tube and stored in liquid nitrogen until use. Equal protein aliquots of lysates were subjected to SDS-PAGE at 40 mA in a discontinuous gel system under reducing conditions (Laemmli, 1970). Resolved proteins were transferred electrophoretically to a nitrocellulose membrane in a Trans-Blot cell (BioRad, Richmond, California) for 18 hr at 40 mV. Excess binding sites were blocked in PBS containing 4% blocking reagent, and the membranes were reacted with anti–flotillin-1 antibody for 1 hr at room temperature. The blots were washed 3 times in PBS, incubated with peroxidase-conjugated goat anti-mouse IgG antibody, and washed; immunoreactive bands were visualized with diaminobenzidine substrate (Sigma).

Statistical analysis

Differences between means for multiple comparison purposes were assessed by Duncans new multiple range test, with statistical significance inferred at $P < 0.05$.

RESULTS

Infectivity of monensin-sensitive and -resistant *E. tenella* sporozoites

There was no difference in the ability of monensin-resistant sporozoites to infect CPKCs following preincubation with PBS, compared with the drug-sensitive parent strain (Fig. 1). As expected, however, infectivity of the monensin-sensitive sporozoites was significantly reduced after monensin treatment (23.2% infectivity), whereas the monensin-resistant parasites were resistant to drug treatment (82.4% infectivity).

Infectivity of monensin-resistant and -sensitive sporozoites was significantly decreased after treatment with the anti–flotillin-1 antibody as compared with that of the sporozoites that were treated with normal IgG (Fig. 2).

Immunofluorescence and immunoblot analysis of flotillin-1 in *E. tenella* sporozoites

Monesin-resistant sporozoites treated with the drug displayed flotillin-1 staining in discrete patches with prominent staining in the apex of the microorganisms (Fig. 3A). The same staining pattern was seen when the monensin-resistant sporozoites were treated with PBS, or the drug-sensitive sporozoites were treated with PBS (Fig. 3B). By contrast, in monensin-sensitive sporozoites treated with the drug, flotillin-1 was more uniformly and diffusely distributed on the parasite surface (Fig. 3C).

Flotillin-1 was present only in the light-density fractions of monensin-resistant sporozoites treated with PBS or monensin (Fig. 4). The same pattern was evident from the monensin-

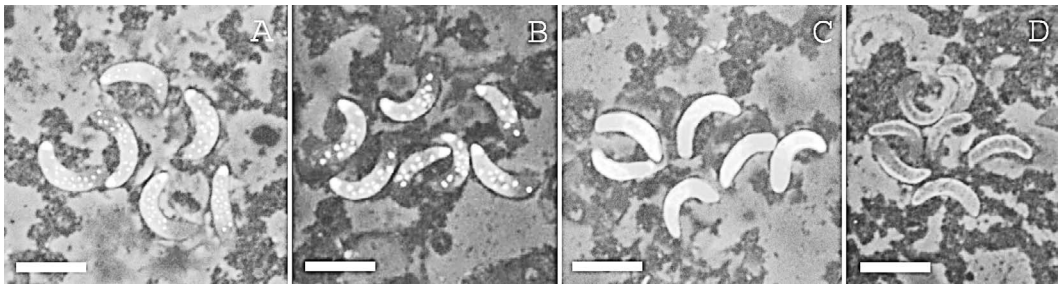

FIGURE 3. Effect of monensin on flotillin-1 expression by *E. tenella* sporozoites. (**A**) Sporozoites of the monensin-resistant strain incubated with monensin, stained with anti–flotillin-1 antibody, and examined by immunofluorescence microscopy. (**B**) Sporozoites of the monensin-sensitive parent strain incubated in PBS. (**C**) Sporozoites of the parent strain incubated with monensin. (**D**) Sporozoites of the parent strain incubated with monensin and stained with normal IgG (negative control). Bar = 10 μm.

sensitive parent strain treated with PBS. However, treatment of the parent strain with monensin resulted in mobilization of flotillin-1 to the nonraft higher-density fractions. Collectively, the results shown in Figures 3 and 4 strongly suggested that monensin disrupts lipid rafts on *E. tenella* sporozoites.

DISCUSSION

The data presented in this report demonstrate that the anticoccidial drug monensin blocks *E. tenella* infectivity of chicken cells in vitro through a mechanism involving, at least in part, the drug's ability to disrupt sporozoite lipid rafts. Monensin is a monovalent ion-selective ionophore that facilitates the transmembrane exchange of, principally, sodium ions for protons. One consequence of this exchange is the neutralization of acidic intracellular compartments such as Golgi cisternae and associated elements, lysosomes, and certain endosomes (Mollenhauer et al., 1990). Monensin inhibits sphingolipids from incorporating into plasma membranes by blocking their transfer to the acid-neutralized Golgi sacs (Gordon and Lloyd, 1994). As a consequence, rafts do not form and raft-associated proteins become uniformly distributed in the membrane.

In the absence of monensin treatment of the parent *E. tenella* strain, or drug treatment of the monensin-resistant strain, flotillin-1 was seen to be highly enriched in the apical region of the parasite, a region that is known to mediate host cell invasion. Flotillin-1, and its homologue flotillin-2, also known as reggie-2 and reggie-1, respectively (Volonte et al., 1999), are encoded by evolutionarily conserved genes with orthologues in mice, rats, humans, fishes, and fruit flies (López-Casas and del Mazo, 2003). Both proteins show molecular similarities and antibodies against one often are cross-reactive with the other (Kokubo et al., 2000). Flotillin-1 consists of 427 amino acid residues with a predicted molecular weight of 48.0 kDa, whereas flotillin-2 possesses a molecular weight of 42.0 kDa (Edgar and Polak, 2002). Thus, although the anti–flotillin-1 antibody used in this study may react with flotillin-2, the size of the protein detected by immunoblotting indicates that it recognized flotillin-1.

The ability of monensin to inhibit *E. tenella* infection of host cells and to redistribute flotillin-1 away from the sporozoite apex suggests that this raft protein is somehow involved in invasion. Upon contact of the sporozoite with the host cell surface, a signal is transduced from the surface to the apex that induces parasite reorientation, microneme exocytosis, apical binding to the host cell, and formation of the parasitophorous

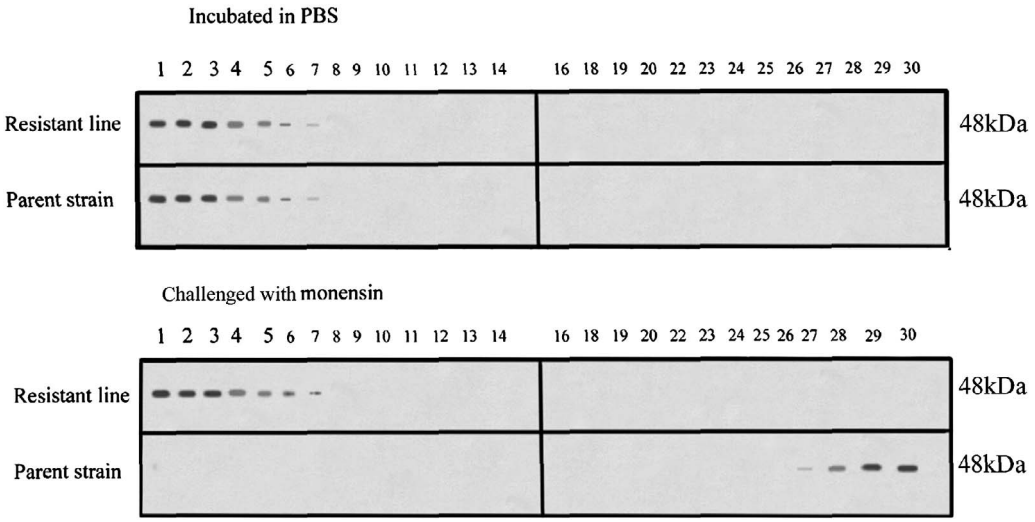

FIGURE 4. Effect of monensin on distribution of flotillin-1 in lipid rafts. Sporozoite membrane lysates from monensin-resistant and -sensitive parent strains were fractionated by sucrose density-gradient centrifugation and individual fractions analyzed by immunoblotting with anti–flotillin-1 antibody. Fraction numbers from the light density (upper, no. 1) to the high density (lower, no. 30) are given at the top of each blot.

vacuole (Dubremetz et al., 1998). Thus, it is apparent that multiple events are involved in the invasion process, including signal transduction, cell migration, organization of the cytoskeleton, protein sorting, and membrane trafficking. It has been proposed that flotillin-1 plays a role in all of those processes (Edidin, 2003). In addition, our observation that anti-flotillin-1 antibody significantly inhibited sporozoite infectivity provides additional evidence that flotillin-1 is involved in *E. tenella* invasion. It is tempting to speculate that the interaction of flotillin-1 with the host cell surface triggers parasite intracellular signaling events leading to activation of the conoid apparatus and the subsequent invasion of sporozoites into host cells.

Although flotillin-1 is known to be a key structural component and marker of lipid rafts, visualization of rafts is hindered by the facts that they contain few resident proteins and are relatively small in size (estimated to be 70 nm or less in dimension) (Schlegel and Lisanti, 2001). Using flotillin-1 as a visualization marker, rafts have been shown to exhibit a patchy, heterogeneous pattern on cells of higher metazoans (Kokubo et al., 2003). In the current study, we observed a similar patchy pattern of flotillin-1 expression on the protozoan sporozoite surface that was disrupted by monensin in the parent parasite strain, but not in the monensin-resistant variant. Combined with the immunoblot data of sucrose density-gradient fractionated sporozoite membranes, these results suggest that the drug is able to destroy the structure of the rafts in parasite membranes.

Because of monensin's ability to integrate into plasma membranes and disrupt its lipid composition, it has been proposed that ionophore resistance is mediated by the ability of the cell to exclude the drug from the membrane. In the case of bacteria, development of monensin resistance was correlated with the ability to produce extracellular polysaccharides that was controlled by proteins encoded by a large number of inducible genes (Roberts, 1996; Russell and Houlihan, 2003). In contrast, the genetic differences between ionophore-sensitive and -resistant strains of *E. tenella* are relatively small and little is known about the genetics of extracellular polysaccharide production by *E. tenella* (Shirley and Bumstead, 1994). Current studies in our laboratory are directed at elucidating the molecular mechanisms by which coccidian parasites develop monensin resistance vis-à-vis the roles of flotillin-1 and lipid rafts in host invasion.

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